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Unusual usage of wobble modifications in mitochondrial tRNAs of the nematode *Ascaris suum*

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Abstract To understand the decoding property of nematode mitochondrial tRNAs with unusual secondary structures, post-transcriptional modifications at wobble positions of *Ascaris suum* mitochondrial tRNAs corresponding to two-codon families ending with a purine were analyzed. 5-Carboxymethylaminomethyluridine ($cmnm^5U$) was identified at the wobble positions of $tRNA^{Lys}$, $tRNA^{Glu}$ and $tRNA^{Gln}$, while 5-carboxymethylaminomethyl-2-thiouridine ($cmnm^5s^2U$) was present in $tRNA^{Leu}_{UAA}$ and $tRNA^{Trp}$. In most bacterial and mitochondrial tRNAs, the 2-thiouridine derivative is present in tRNAs for Lys, Glu and Gln. These is no report that $cmnm^5s^2U$ is used in $tRNA^{Leu}_{UAA}$ and $tRNA^{Trp}$. The unusual usage of wobble modifications might assist decoding of nematode mitochondrial mRNAs.
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Keywords: Modified nucleoside; Nematode; Mitochondrial tRNA; T-armless tRNA; Wobble position; 2-Thiouridine

1. Introduction

Post-transcriptional modifications are known to play an important role in tRNA function and structure. Modified nucleosides at the wobble position are required for accurate decoding of genetic information. The wobble position of the tRNA responsible for decoding all four codons in a four-codon family box is mostly occupied by unmodified uridine [1], whereas the wobble uridine of the tRNA corresponding to two-codon families ending with a purine (NNR) is usually modified to discriminate cognate codons from near-cognate codons [2,3]. Those uridines at the wobble position that are modified with a C5 substituent or its 2-thio derivative have been characterized in detail [4–13].

In animal mitochondrial (mt) tRNAs, unique modifications have been found at the wobble position. Recently, taurine-containing modified uridines (5-taurinomethyluridine, τm^5U, and 5-taurinomethyl-2-thiouridine, τm^5s^2U) were identified in mammalian mt tRNAs corresponding to NNR codons [14]. The lack of these modifications causes certain mitochondrial diseases in humans [15,16]. Such taurine-containing modifications have also been found in urochordate mt $tRNA^{Gly}_{UCU}$ [17] (Kondow et al., unpublished observations). However, in yeast [18] and *Tetrahymena* [2], 5-carboxymethylaminomethyluridine ($cmnm^5U$) derivatives are present at the wobble position of mt tRNAs corresponding to the two-codon families. We wondered when during the evolution from single-celled eukaryotes to mammals the 5-carboxymethylaminomethyl group was replaced with the 5-taurinomethyl group in mitochondria, with particular interest in the nematode.

Nematode mt tRNAs have extremely unusual secondary structures in that they lack the T-arm or D-arm [19–22] found in the cloverleaf secondary structures of canonical tRNAs [2]. To understand how these tRNAs function in the nematode mt translation system, a characterization of their post-transcriptional modifications is first of all necessary. To date, 15 tRNAs (13 T-armless tRNAs and 2 D-armless $tRNA^{Ser}$) have been characterized [23–25] in *A. suum* mitochondria. In a previous study, we reported that all T-armless *A. suum* mt tRNAs examined had 1-methyladenosine at position 9 (m^1A_9) and that this modification was indispensable for their structure, aminoacylation, and EF-Tu-binding [25]. These observations exemplify the importance of post-transcriptional modifications for tRNA structure and function.

Within the context of these previous studies, we attempted in the present study to identify the modifications at wobble positions of nematode mt tRNAs corresponding to NNR codons by isolating and determining the RNA sequences of tRNAs from the nematode *A. suum*. Here we report the characteristic wobble modifications of the *A. suum* tRNAs.

2. Materials and methods

2.1. Purification of individual mt tRNAs from A. suum

Ascaris suum were provided by Dr. K. Kita and Dr. Y. Watanabe (Univ. of Tokyo). The crude RNA fraction was extracted from 200 g of *A. suum* as described [23]. Individual mt tRNAs were purified by

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Abbreviations: A. suum, *Ascaris suum*; APM, (*N*-acryloylamino)phenyl-mercuric chloride; $cmnm^5U$, 5-carboxymethylaminomethyluridine; m^1A, 1-methyladenosine; mt, mitochondrial; NNR codons, two-codon families ending with a purine; τm^5U, 5-taurinomethyluridine; τm^5s^2U, 5-taurinomethyl-2-thiouridine; $cmnm^5s^2U$, 5-carboxymethylaminomethyl-2-thiouridine; 2D-TLC, two-dimensional thin layer chromatography; U_{34}, uridine at position 34

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chaplet column chromatography [14], which was invented for tRNA isolation based on a hybrid selection method using solid-phase DNA probes [26]. The sequences of the probes were as follows: $tRNA^{Leu}_{UAA}$, 5′-AGTTGTCCCATATCTTTTACGCTTAAAACA-3′; $tRNA^{Trp}$, 5′-TGAAAACCAAGAGTTTAAACTTAACTTAAA-3′; $tRNA^{Gln}$, 5′-CTATACTACAACCCTTTTACACCAAAAATA-3′; $tRNA^{Lys}$, 5′-AAAAATCTAACACTTTAAACTTAAGTTAAC-3′; $tRNA^{Glu}$, 5′-CGAAAAAGAAATATACAAAAAATTTATACT-3′.

2.2. Sequencing of mt tRNAs

5′- or 3′-^{32}P labeled *A. suum* mt tRNAs were purified again by gel electrophoresis and sequenced by the method of Donis-Keller [27] using RNases T_1, U_2, PhyM, and CL_3.

2.3. Two-dimensional thin layer chromatography

The nucleotide sequences of $tRNA^{Leu}_{UAA}$, $tRNA^{Trp}$, $tRNA^{Gln}$, $tRNA^{Lys}$, and $tRNA^{Glu}$, including modified nucleotides, were analyzed by two-dimensional thin layer chromatography (2D-TLC) as previously described [28].

2.4. Liquid chromatography–mass spectrometry

For nucleoside analysis, each tRNA was purified by PAGE before nuclease digestion. Nucleosides obtained from digestion of mt tRNAs with nuclease P_1 and bacterial alkaline phosphatase were analyzed by LC/MS as described [14,29]. Chromatography was carried out using an ODS reversed-phase column under the conditions reported in [29]. Oligoribonucleotides produced by RNase T_1 digestion of each tRNA were also analyzed by LC/MS as described in the literature [30,31]. For chromatography, the hydrolysates were applied directly onto a Supelcosil LC-DB C_{18} column (300 × 1.0 mm; Supelco), and separated using a 33-minite gradient of 5–80% solvent B (50% methanol) in solvent A (0.4 M 1,1,1,3,3,3-hexafluoro-2-propanol adjusted to pH 7.0 with triethylamine).

2.5. Phenyl-mercuric gel electrophoresis

The presence of the 2-thio derivative in mt tRNAs for Leu(UUR) and Trp was verified by electrophoresis using a 10% polyacrylamide gel containing 7 M urea and 0.015 mg/ml (*N*-acryloylamino)phenylmercuric chloride (APM) [32], which was provided by Dr. N. Shigi and Mr. Y. Yamamoto in our laboratory.

3. Results

3.1. Purification and sequencing analysis of mt tRNAs

We isolated mt tRNAs for Leu(UUR), Trp, Gln, Lys, and Glu, all of which read NNR codons. The sequences of these tRNAs were analyzed by the methods of Donis-Keller [27] (Fig. S1) and Kuchino et al. [28] (Fig. S2). We also analyzed nucleosides obtained by nuclease P_1 digestion of each tRNA by LC/MS (Fig. 1 and S3). These analyses resulted in the tRNA sequences shown in Fig. 3. Modified nucleosides in the wobble position of these five tRNAs were compared with other organisms as shown in Table 1.

3.2. Wobble position corresponding to NNR codons

All of the tRNAs which read NNR codons had $cmnm^5U$ or its 2-thio derivative at the wobble position, but the τm^5U derivative that has been found in mammalian mt tRNAs [14] was not detected. Unexpectedly, $cmnm^5s^2U_{34}$ was detected in $tRNA^{Leu}_{UAA}$ (Fig. 1A) and $tRNA^{Trp}$ (Fig. S3[A]) but not in tRNAs for Lys, Gln, or Glu, which have $cmnm^5U_{34}$. The presence of these modifications was also confirmed by LC/MS analysis of anticodon-containing fragments obtained by RNase T_1-digested tRNAs for Leu(UUR) and Lys (Fig. 1C–F). As for $tRNA^{Leu}_{UAA}$ and $tRNA^{Trp}$, not only $cmnm^5s^2U_{34}$ but also $cmnm^5U_{34}$ was detected by LC/MS analysis (Fig. 1A, C and E for $tRNA^{Leu}_{UAA}$; Fig. S3[A] for $tRNA^{Trp}$), indicating that these are partial modifications. On the basis of the UV chromatogram (Fig. 1 and S3) and the enzymatic digestion pattern obtained by Donis-Keller's method (Fig. S1), 90% of the $cmnm^5U_{34}$ derivatives in $tRNA^{Leu}_{UAA}$ were found to be the 2-thio modification variant. On the other hand, $tRNA^{Trp}$ consists of three different species that contain 35% $cmnm^5U$, 15% $cmnm^5s^2U$, and 50% unmodified uridine, respectively.

3.3. Phenyl-mercuric gel electrophoresis

The presence of $cmnm^5s^2U$ was further confirmed by electrophoresis in gels containing APM [32]. In this analysis, the mercuric compound in the gel interacts specifically with tRNAs containing a thiocarbonyl group, which retards the migration of these tRNAs. Specific retardation of $tRNA^{Leu}_{UAA}$ and $tRNA^{Trp}$ on the APM gel (Fig. 2) indicated the presence of a thiocarbonyl group in these tRNAs.

4. Discussion

In this study, we analyzed wobble modifications of tRNAs corresponding to NNR codons to evaluate when the τm^5 group replaced the $cmnm^5$ group during the evolution of single-celled eukaryotes to mammals. We found that the $cmnm^5$ group is used in nematode mt tRNAs, and that some of them possess a characteristic 2-thio modification of the wobble uridine.

The $cmnm^5$ group in the wobble uridine of mt tRNAs may have been replaced with τm^5 group during evolution, as shown in Table 1. Apart from the 2-thio modification or 2′-*O*-methylation, $cmnm^5U_{34}$ derivatives exist in eubacteria; mitochondria of unicellular eukaryotes, such as yeast and *Tetrahymena* ($cmnm^5U_{34}$ in $tRNA^{Trp}$) [2], and nematode mitochondria, while taurine-containing modifications at U_{34} exist in mitochondria of higher animals such as vertebrates [14] and urochordates [17] (Kondow et al., unpublished observations). Thus, the evolutionary boundary between $cmnm^5U_{34}$ and τm^5U_{34} use in mt tRNAs is most likely between nematodes and urochordates.

2-Thiouridine (s^2U_{34}) derivatives at the anticodon wobble position have been reported in tRNAs for Gln, Lys, and Glu from various sources, including eubacteria and eukaryotic cytoplasm, mitochondria, and chloroplasts, but have never been found in tRNAs for Leu(UUR) and Trp [2,3] (Table 1). In the present study of *A. suum* mt tRNAs, an s^2U_{34} derivative was found in $tRNA^{Leu}_{UAA}$ and $tRNA^{Trp}$. In contrast, there was no s^2U derivative in *A. suum* mt $tRNA^{Gln}$, $tRNA^{Lys}$, or $tRNA^{Glu}$, although in eubacteria these tRNA species usually have an s^2U_{34} derivative [2,3]. Why do nematode mt tRNAs use this s^2U_{34} derivative? It is possible that the 2-thio group of the wobble uridine in *A. suum* mt tRNAs is necessary to raise the efficiency of translating NNR codons. UUR codons appear quite frequently in nematode mt protein genes, and have been found to comprise 12–13% of all codons [20,33]. This value is much higher than the average usage of other two-codon families (about 3%). Thus, translation errors involving UUR codons would result in serious consequences for mt protein synthesis. In addition, UUY codons specifying phenylalanine also occur frequently (13–15%) [20], so that it is very important for the nematode mt translation system to avoid the misreading of $tRNA^{Leu}_{UAA}$ against UUY codons. The 2-thio modification leads to an increase in the 3′-endo conformation of the

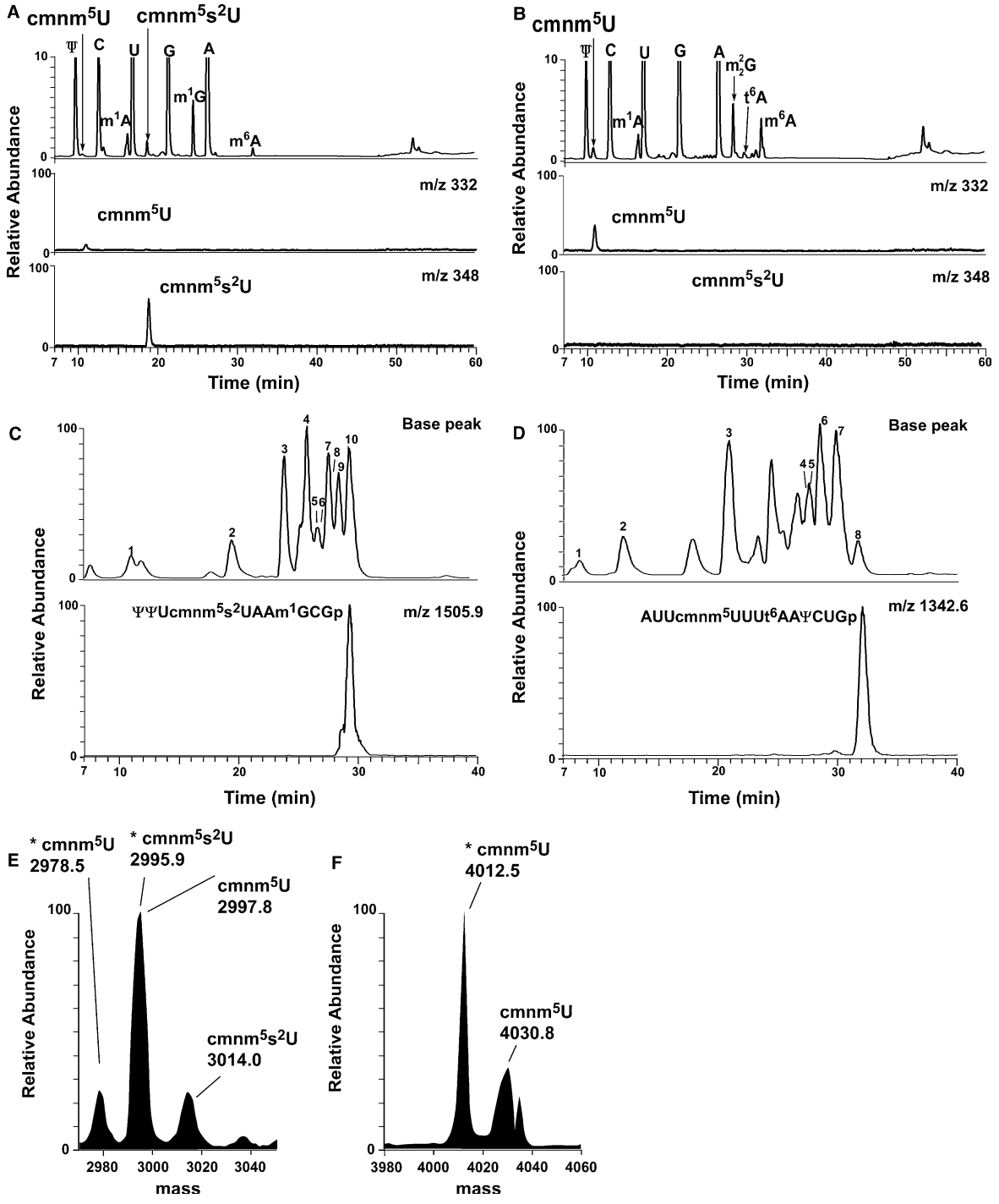

Fig. 1. LC/MS analysis of *A. suum* mt $tRNA^{Leu}_{UAA}$ and $tRNA^{Lys}$. (A, B) Total nucleoside analysis of purified *A. suum* mt $tRNA^{Leu}_{UAA}$ and $tRNA^{Lys}$, respectively. (top) UV chromatograms for nucleosides. (bottom) Mass chromatograms for $cmnm^5s^2U$ and $cmnm^5U$ with mass filters at *m/z* 332 and 348. The relative peak intensity of $cmnm^5s^2U$ and $cmnm^5U$ in the mass chromatograms are adjusted against that of m^1A as the standard (100%). (C, D) Chromatograms for RNase T_1-digested $tRNA^{Leu}_{UAA}$ and $tRNA^{Lys}$, respectively. (top) UV traces at 260 nm; numbers indicate elution positions of RNA fragments as follows: (C) 1, UGp; 2, UUGp; 3, AAGp; 4, $UUAUm^1AGp$; 5, AUAUGp; 6, CAΨΨUGp; 7, CAUAAGp; 8, ΨAAAAGp; 9, ACAACUCCA; 10, $ΨΨUcmnm^5s^2UAAm^1GCGp$. (D) 1, CCA; 2, UGp; 3, UUGp; 4, AAAUGp; 5, $Ψm^2_2GΨΨAGp$; 6, UUUAAAGp, 7, $UUm^1ACUUAAGp$; 8, $AUUcmnm^5UUUt^6AAΨCUgp$. (middle) Base peak mass chromatograms. (bottom) Mass chromatograms for *m/z* 1505.9 ($tRNA^{Leu}_{UAA}$) and *m/z* 1342.6 ($tRNA^{Lys}$) to detect doubly charged and triply charged ions of anticodon-containing fragments, respectively. (E, F) Deconvoluted mass spectra for anticodon-containing fragments derived from $tRNA^{Leu}_{UAA}$ and $tRNA^{Lys}$, respectively. The calculated *m/z* values are given for each peak. The peaks of fragments with 3′-cyclic ends are shown with asterisks.

uridine nucleotide [4,34]. Thus, the 2-thio modification of U_{34} has been considered to be involved in the rigidity of the anticodon structure [4–7], which restricts the tRNA to recognizing NNR codons and not NNY codons [11,12]. Furthermore, this modification has been shown to contribute to aminoacylation efficiency [8,9] and the translation rate [10,13]. The 2-thio

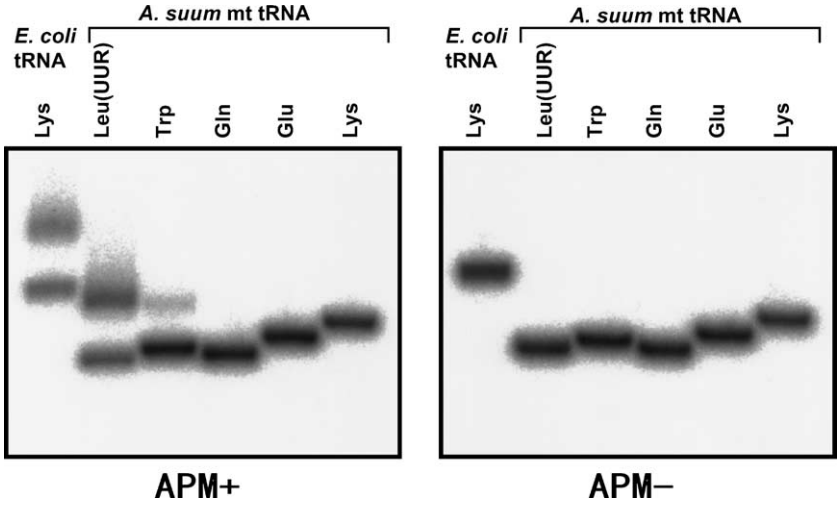

Fig. 2. Phenyl-mercuric gel electrophoresis of mt tRNAs to detect specific retardation due to the thiocarbonyl group. 5′-Labeled tRNAs were analyzed on denaturing 10% polyacrylamide gels with (left, APM+) or without (right, APM−) APM.

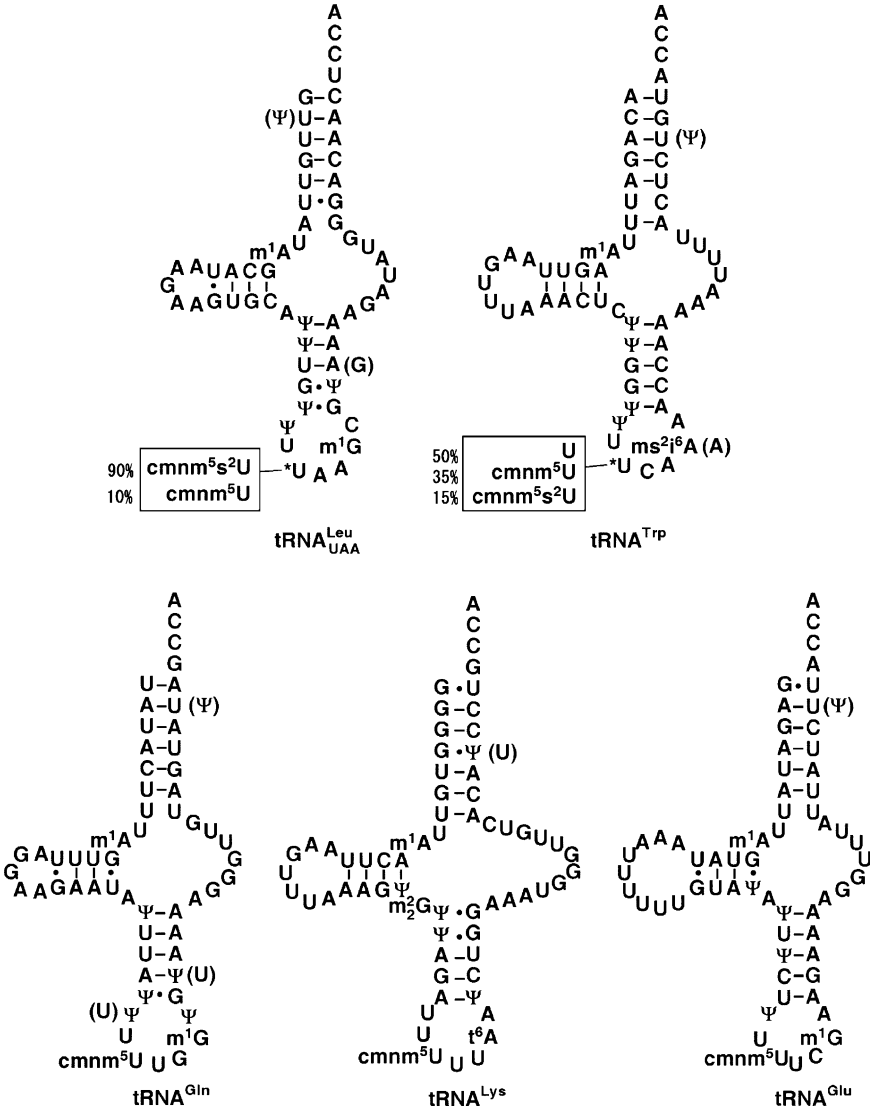

Fig. 3. Nucleotide sequence and secondary structure of *A. suum* mt $tRNA^{Leu}_{UAA}$, $tRNA^{Trp}$, $tRNA^{Gln}$, $tRNA^{Lys}$, and $tRNA^{Glu}$. The letters in the parentheses indicate partial modification or genetic polymorphism.

Table 1
Modified nucleosides in the wobble position of tRNA

	UYR (Y = U or C, R = A or G)		VAR (V = C, A or G, R = A or G)		
	Leu(UUR)	Trp	Gln	Lys	Glu
Nematode mt					
A. suum	$cmnm^5s^2U$, $cmnm^5U$	U, $cmnm^5U$, $cmnm^5s^2U$	$cmnm^5U$	$cmnm^5U$	$cmnm^5U$
Eubacteria					
E. coli	$cmnm^5Um$ [35]	(inexistent)	s^2U^* [36]	mnm^5s^2U	nm^5s^2U
M. capricolum	$cmnm^5U$	$cmnm^5Um$	$cmnm^5U$	$cmnm^5U$	$cmnm^5U$
Fungous mt					
S. cerevisiae	$cmnm^5U$ [18]	$cmnm^5U$ [18]		$cmnm^5U^*$ [18]	
Vertebrate mt					
Bovine	τm^5U [14]	U*		τm^5s^2U [14]	U*
Human	τm^5U [14]	U*		τm^5s^2U [14]	U*

Blanks indicate unidentified nucleosides. Numbers in parentheses denote references, and modified nucleotides without parenthesis are referred to http://www.uni-bayreuth.de/departments/biochemie/trna/ [2] except for those of *A. suum* mt tRNAs. Asterisks show unknown derivatives of the indicated modified nucleosides.

group at wobble uridine in *A. suum* mt $tRNA^{Leu}_{UAA}$ probably raises translation efficiency and specificity for UUR codons to prevent the possible errors by discriminating from UUY codons. The other two-codon sets, CAR (Gln), AAR (Lys), GAR (Glu), and UGR (Trp), are not used frequently (1.2–3.2% in mt protein genes of nematode) [20]. This might explain why tRNAs for Gln, Lys, and Glu do not have a 2-thio modification and why $tRNA^{Trp}$ has only a partial 2-thio modification.

In conclusion, we found unusual use of the s^2U_{34} derivative in nematode mt tRNAs, which might raise the translation efficiency of frequently used codons. We also found that nematode mt tRNAs for NNR codons have the $cmnm^5$ group at the wobble uridine. The τm^5 modification of the wobble uridine, which is related to human mitochondrial disease, was generated probably during the course of evolution between nematodes and urochordates. Further analysis of the functional differences between τm^5 and $cmnm^5$ groups and the co-evolution of the ribosome site responsible for codon–anticodon interaction during the change from $cmnm^5$ to τm^5 should shed further light on the biological meaning of this evolution.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version at doi:10.1016/j.febslet.2005.04.009.

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